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(54) Title: SCREENING METHODS EMPLOYING FISH MODEL ASSESSMENT OF VISION

(57) Abstract: Fish such as zebrafish are provided and used in a suppressor screen or other screen to identify a gene or test substance that affects activity or effect of a second gene provided transgenically that affects vision of the fish, or a treatment of the fish that affects vision. Vision of model fish is compared with vision of mutated or treated fish, allowing for identification of genes and test substances with therapeutic application.

SCREENING METHODS EMPLOYING FISH MODEL ASSESSMENT OF VISION

The present invention relates to generation and use of disease
5   models, for instance in assays to identify and investigate genes
and substances involved in disease and disease treatment,
identification and use of drug targets. In particular, the
present invention relates to generation and use of disease models
in fish such as zebrafish. The invention further relates to use
10   of fish, e.g. zebrafish, disease models in genetic suppressor
screens.

The genes responsible for numerous diseases are now known. In
many cases, animal models have been created for these diseases.
15   Most such models are in mammals, such as rats and mice, or in
invertebrates such as *Drosophila*. The advantage of using
mammalian models is their greater similarity to humans, while the
use of invertebrates offers the opportunity to perform
sophisticated genetic analysis to identify genes involved in
20   disease.

While the identification of disease causing genes is useful in
helping elucidate the mechanisms of disease, it does not
necessarily lead to strategies for treatment. It would be very
25   useful to have a model system in which screens can be carried out
to identify a gene to cure a previously existing disease. Such a
system would be particularly relevant to human disease if
performed in a vertebrate and *in vivo*.

30   The zebrafish is an organism which combines many of the
advantages of mammalian and invertebrate model systems. It is a

2

vertebrate and thus more relevant in models of human disease than
Drosophila or other invertebrates, but unlike other vertebrate
models it can be used to perform genetic screens.

5   The zebrafish has a short maturation period of two to three
     months and is highly fecund, with a single pair of adults capable
     of producing 100 to 200 offspring per week. Both embryos and
     adults are small, cheap and easy to maintain, offering the
     potential of large scalability. It is possible to introduce
10   random mutations into the zebrafish genome, for example with the
     use of chemical mutagenesis (Solnica-Krezel et al., Genetics
     1994, 136(4): 1401-20). It is also possible to make transgenic
     fish carrying exogenous genes. Zebrafish expressing a
     heterologous Ikaros protein has been used to model haematopoiesis
15   and lymphoproliferative disorders (WO 01/40273).

     WO99/42606 concerns a method of screening an agent for an
     angiogenesis activity or cell death activity or toxic activity,
     comprising administering the agent to a teleost (e.g. zebrafish,
20   medaka, Giant rerio or puffer fish), and detecting a response in
     the teleost indicating angiogenesis activity or an effect on cell
     death activity or toxic activity in at least one tissue or organ
     of the teleost.

25   WO01/12667 describes use of a transgene to drive marker
     expression in the eye. The organism may be fish. It suggests
     making a transgenic animal (which it says may be a fish) by a
     method comprising introducing a genetic construct for expression
     of a marker sufficient to visually detect the marker in
30   photoreceptive cells or organ and selecting for transgenesis by
     visually detecting the marker in a photoreceptive cell or organ.

WO01/51604 (Exelixis) is concerned with providing sensitizer
genes such as a tumor gene or an oncogene in a non-human animal
(for which zebrafish are mentioned as a passing, hypothetical
5   possibility) in cells where expression is non-lethal. It is
proposed to detect changes and compare on mutation or other
treatment. The aim is identification of "interactor genes" that,
when mutated, specifically kill or reduce the size of target
tissue (subject to the sensitizer gene).

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Kennedy et al. J Biol Chem (2001) 276, 14037-14043 looks at
expression of a marker gene such as GFP in the eye of zebrafish
under control of a zebrafish rod opsin promoter.

15   WO98/56902 discloses use of transgenic fish, including zebrafish,
and methods of crossing fish strains, including strains with
mutations, the aim being to identify genes that affect expression
of fish genes.

20   In addition to Zebrafish, other fish such as fugu, goldfish,
medaka and giant rerio are amenable to manipulation, mutation and
study, and use in aspects and embodiments of the present
invention as disclosed herein.

25   The present invention provides fish (e.g. zebrafish) disease
models not only representative of the underlying disease but also
amenable for use in a subsequent screen. Advantageously, the
present invention allows for study and screening where the
disorder or disease would without the invention result in fish
30   not likely to be viable nor able to breed. The invention is
generally applicable to any of a variety of diseases and

4

disorders, and a range of examples is specifically set out
herein.

The present invention provides means, specifically transgenic
5   fish such as zebrafish, for use in methods of screening for and
identifying a gene which, when mutated, alters the activity or
effect of a disease gene and thus a disease phenotype. A
secondary gene of which mutation affects activity or effect of a
primary disease gene is termed a suppressor gene. Suppressor
10   genes represent targets for drugs to treat the disease.

In preferred embodiments of the present invention, a gene,
especially a disease gene, is introduced into a fish, e.g.
zebrafish, under regulatory control of a promoter that is eye-
15   specific. Thus, genetic modification to introduce a disease gene
which would normally lead to animal lethality, lack of viability
and/or lack of reproductive ability is effected in such a way
that lethality, lack of viability and/or lack of reproductive
ability is avoided. For screening purposes it is highly
20   advantageous that the mutated or genetically-modified subject
organism is able to reach reproductive age and to generate new
offspring also harbouring the genetic alteration.

In other embodiments, a transgenic model fish may be treated with
25   a test substance to screen for a test substance able to affect
activity or effect of the transgene, by assessment of vision of
the fish, and/or may be subject to mutation to identify a gene in
the fish that affects activity or effect of the transgene on
vision. Similarly, a model fish may be generated by treatment
30   with a substance, e.g. with a substance that affects vision such
as 2,5 hexandedione (arch toxicol 1998: 72: 597-600), N-3--

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pyridylmethyl-N'-p-nitrophenylurea (Br J Ophthalmol 1988 78:584-
90), or a physical treatment, such as light: light induced
degeneration has also been described (J Neuobiol 2000 44: 289-
307). A model fish provided by means of such a treatment may
5   then be used in a screen for a gene that affects the effect of
the treatment and/or for a test substance that affects the effect
of the treatment. Effect of a gene or a treatment may be
assessed by comparing vision of model and treated and/or mutated
fish.

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In one aspect, the present invention provides a method of
screening for a substance or gene (termed herein "first gene")
that affects activity or effect of a second gene, or activity or
effect of a treatment, on a fish, the method comprising:
15   providing, as model fish for screening, (i) fish transgenic
for the second gene, wherein the second gene is under regulatory
control of an eye-specific promoter and expression of the second
gene within the eye of the fish affects vision of the fish, or
(ii) fish subject to said treatment, wherein the treatment
20   affects vision of the fish;
           mutating said model fish to provide mutated fish or treating
said model fish with a test substance to provide treated fish;
           comparing vision of mutated fish or treated fish with vision
of model fish in order to identify any mutated fish or treated
25   fish with altered vision compared with model fish;
           thereby to identify a test substance that affects activity
or effect of the second gene or activity or effect of said
treatment, or, by identifying a genetic difference between model
fish and mutated fish with such altered vision, to identify a
30   first gene that affects activity or effect of the second gene or
activity or effect of said treatment.

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The method may comprise mutating model fish transgenic for the second gene to provide mutated fish and identifying a first gene that affects activity or effect of the second gene.

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The method may comprise treating with a test substance model fish transgenic for the second gene to provide treated fish and identifying a test substance that affects activity or effect of the second gene.

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The method may comprise mutating model fish subject to said treatment to provide mutated fish and identifying a first gene that affects activity or effect of said treatment.

15   The method may comprise treating with a test substance model fish subject to said treatment to provide treated fish and identifying a test substance that affects activity or effect of said treatment.

20   The method may comprise identifying a first gene that lessens activity or effect of the second gene.

The second gene may be a disease gene.

25   The method may comprise identifying a first gene that enhances or increases activity or effect of the second gene.

The method may comprise identifying a test substance that lessens activity or effect of the second gene or said treatment.

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The second gene may be a disease gene.

The method may comprise identifying a test substance that enhances activity or effect of the second gene or said treatment.

5   Fish may be treated with a substance in a number of ways. Fish may be contacted with a test substance, it may be touched or rubbed on their surface or injected into them. A test substance may be added to water in which they are. A different test substance may be added to each well of a multi-well plate, such

10   as a 96 well plate, to identify that test substance exhibiting a beneficial or deleterious effect. There may be one or multiple fish in each well exposed to the test substance. The test substance may be added prior to the onset of the disease phenotype, concurrent with the onset of the disease phenotype, or

15   subsequent to the onset of the disease phenotype. The same test substance may be added to different wells at different concentration. For example, test substance 1 may be added to well A1 at a concentration of 1mM, to well A2 at a concentration of 100uM, to well A3 at a concentration of 10uM, to well A4 at a

20   concentration of 1uM and to well A5 at a concentration of 0.1uM. Then test substance 2 to well B1 etc. The panel of test substances may be known drugs or new chemical entities.

Additionally, the test substances may be added in combination.
25   For example, well A2 may contain test substance 1 and 2, well A3 test substance 1 and 3, well B2 test substance 2 and 3. Alternatively, every well may contain test substance x, with individual wells containing a panel of additional test substances.

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In other options, a population of fish in a petri dish or a tank
may be employed and treated together, e.g. via addition of one or
more or a combination of test substances in the water.

5   A diverse library of drug-like compounds, such as the LOPAC
     library (Sigma) may be used, or the Chembridge PHARMACOphore
     diverse combinatorial library. Other targeted libraries against
     particular targets classes may be used, such as ion channel
     libraries or G protein libraries. The latter is of particular
10   relevance for vision, given that the phototransduction cascade is
     the archetypal G protein cascade.

     The following drugs are examples that may have some synergistic
     action together: sodium valproate, ethosuximide, phenytoin,
15   carbamazepine, lamotrigine, gabapentin, phenobarbitone, diazepam,
     clobazam, tiagabine and levetericatem. Any combination of these
     and/or other drugs or test substances may be arrayed in every
     possible combination and tested for synergistic efficacy.
     Appropriate doses vary from 0.1uM to 10mM, with 100uM being a
20   reasonable starting concentration to assess. This will pick up
     beneficial effects, with for example, diazepam showing
     therapeutic effects in monotherapy at 35uM concentration.

     According to one aspect of the present invention there is
25   provided a fish, e.g. a zebrafish, fugu, goldfish, medaka or
     giant rerio transgenic for a disease gene under regulatory
     control of an eye-specific promoter, wherein expression of the
     disease gene within the eye of the fish affects vision of the
     fish.

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The promoter used to control eye-specific expression may be inducible, which may facilitate establishment and/or screening of a fish line.

5   An eye-specific promoter for use in the present invention provides for expression of a disease gene in a component or tissue of the eye involved in one or more of: the passage of a photon of light through the eye, the conversion of photon energy into a biological signal, and the transmission of this signal to
10   the visual centres of the brain. Thus, a promoter for use in the present invention may be functional in one or more of any of the following tissues and cells: conjunctiva, cornea, aqueous humour, vitreous humour, lens, retina, optic nerve, any retinal cell, rod photoreceptor, cone photoreceptor, ganglion cell, glial
15   cell, horizontal cell, bipolar cell and amacrine cell.

Suitable eye-specific promoters are disclosed and discussed elsewhere herein, these being examples, each preferred in an individual embodiment of the invention, while others are
20   available to the skilled person.

In particular embodiments the disease gene when expressed results in a disease phenotype in a dominant fashion. The invention involves placing the gene under the control of an eye-specific
25   promoter, rather than its own natural promoter, to avoid lethality. The disease is then only manifest in the eye, so the fish is blind or at least has impaired vision or visual function compared with wild-type. Visually impaired and blind fish are viable and breed.

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An alternative method involves placing the gene under the control of a promoter which is inducible and thus can be switched off and on at will. The disease process can then be switched off before the fish die.

A disease gene to be employed in an embodiment of the present invention may be any gene in wild-type or mutant form which, when expressed in a fish such as zebrafish, in preferred embodiments in a component of the fish eye, results in abnormal development, dysfunction or degeneration of tissue or cell function, thus when expressed in a component of the eye causes abnormal development, dysfunction or degeneration of a tissue or cell of the eye (e.g. as set out already above), this affecting visual function of the fish. In some embodiments, the expression of the disease gene in a component of the eye causes failure of development or degeneration of a part of the visual system. In other embodiments, the expression of the disease gene in a component of the eye causes a component of the visual system to malfunction.

In one preferred embodiment of the present invention, expression of human alpha-synuclein carrying A53T mutation is expressed in fish, e.g. zebrafish, retinal ganglion cells, leading to a degeneration of the ganglion cells. Transmission of visual information to the brain is consequently impaired, and thus visual function of the fish adversely affected. Fish with alpha-synuclein expressed in their retinal ganglion cells perform more poorly than normal on visual testing.

In another preferred embodiment, human rhodopsin carrying P347S mutation is expressed in fish, e.g. zebrafish, photoreceptors, leading to a degeneration of the photoreceptor cells.

11

Consequently, photons are no longer detected by the retina and thus the vision of the fish is impaired, as determined on visual testing.

5   In preferred individual embodiments, the dominant acting disease gene is selected from the group consisting of huntingtin, alpha-synuclein, presenilin-1, presenilin-2, TNF, SMN and rhodopsin, either in wild type or mutant forms.

10   Accession numbers and references for these genes are as follows, and are all incorporated herein by reference:

Huntingtin: accession for wild type human gene OMIM 143100; Huntington's Disease Collaborative Research Group, *Cell* 72: 971-
15   983, 1993. PubMed ID : 8458085, Marsh et al. *Hum Mol Genet* 2000;9(1):13-25;

Alpha-synuclein: accession for wild type human gene XM003494; Spillantini et al. *Nature* 1997;388(6645):839-40;
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Presenilin-1: accession for wild type human gene AH004968; Sherrington et al. Nature 375 (6534), 754-760 (1995);

Presenilin-2: accession for wild type human gene NM_000447; Levy-
25   Lahad et al. Science 269 (5226), 973-977 (1995), Levy-Lahad et al. Science 269 (5226), 970-973 (1995), Rogaev et al. Nature 376 (6543), 775-778 (1995), Levy-Lahad et al. Genomics 34 (2), 198-204 (1996);

30   TNF: accession for wild type human gene XM_055614; Bitsch et al. Glia. 2000 Feb 15;29(4):366-75, Liu et al. Nat Med. 1998

12

Jan;4(1):78-83, Probert et al. Proc Natl Acad Sci U S A. 1995 Nov
21;92(24):11294-8;

5   SOD-1: accession for wild type human gene AY049787; Rosen et al.
Nature 1993;362(6415):59-62, Parkes et al. *Nat Genet*
1998;19(2):171-4;

SMN: accession for wild type human gene XM_041493; Pellizzoni et
al. *Cell* 1998, 95(5):615-24, Miguel-Aliaga et al. *FEBS Lett*
10   2000;486(2):99-102;

Rhodopsin: accession for wild type human gene U49742, Kaushal et
al. *Biochemistry* 1994;33(20):6121-8, Li et al. *Proc Natl Acad Sci
U S A* 1996;93(24):14176-81, Colley et al. *Proc Natl Acad Sci U S
15   A* 1995;92(7):3070-4.

The coding sequence of a gene may be placed under the control of
(or "operably linked to") the ath5, opsin, rhodopsin or other
promoter that expresses in a tissue or cell of the eye. See for
20   example the following references: Mani et al. (2001) J. Biol.
Chem. 276(39): 36557-65 (rhodopsin), Knox et al. (1998) FEBS
Lett. 423(2): 117-21, Kennedy et al. (2001) J. Biol. Chem. 276
(17): 14037-43 and Quiambao et al. Vis. Neurosci. (1997) 14(4):
617-25 and Kido et al. (1996) Curr. Eye Res. 15(8): 833-44
25   (opsin).

The coding sequence of a gene may be placed under the control of
an inducible promoter, such as a promoter selected from a heat
shock promoter, or a tetracycline or hormone inducible system.
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The invention provides for manipulation of nucleic acid in order
to modify cells of fish such as zebrafish, as disclosed. Nucleic
acid of a disease gene to be expressed in fish in accordance with
the invention is to be integrated into the chromosome of cells.
5   Integration may be promoted by inclusion of sequences which
promote recombination with the genome, in accordance with
techniques available in the art. The disease gene may be
heterologous to the fish, e.g. may be heterologous to zebrafish
(e.g. mammalian, such as human), and may be in wild-type form or
10 in any allelic or mutant form. The disease gene may be a
zebrafish or other fish gene, in wild-type or mutated form, e.g.
to provide an extracopy of a zebrafish or other fish gene, such
as in a mutated disease form.

15 Nucleic acid sequences encoding the peptides or polypeptides of
the present invention may be readily prepared by the skilled
person using the information and references contained herein and
techniques known in the art (for example, see Sambrook and
Russell "Molecular Cloning, A Laboratory Manual", Third Edition,
20 Cold Spring Harbor Laboratory Press, 2001, and Ausubel et al,
Current Protocols in Molecular Biology, John Wiley and Sons,
1992, or later edition thereof). See Detrich et al. (1998) The
Zebrafish: Biology. Methods in Cell Biology. Volume 59, and
Detrich et al. (1998) The Zebrafish: Genetics and Genomics.
25 Methods in Cell Biology. Volume 60 for techniques of zebrafish
maintenance, mutagenesis, transgenesis and mapping.

The desired coding sequence may be incorporated in a construct
having one or more control sequences operably linked to the
30 nucleic acid to control its expression. Appropriate regulatory
sequences, including promoter sequences, terminator fragments,

polyadenylation sequences, enhancer sequences, marker genes and other sequences as appropriate may be included.

Regions responsible for promoter and enhancer activity of a gene
5   known to be expressed in a desirable pattern such as only in the retina, may be isolated by ligating stretches of sequence from upstream of the translation start codon in the gene to a reporter gene. Constructs with deletions in putative promoter and/or enhancer regions are generated and the constructs tested for
10 tissue specific gene expression in transgenic fish, e.g. transgenic zebrafish, fugu, goldfish, medaka or giant rerio.

A selectable marker, for example gene encoding a fluorescent protein such as Green Fluorescent Protein (GFP) may be included
15 to facilitate selection of clones in which the gene construct has inserted into the genome. Where a fluorescent marker is used, embryos may be screened under a fluorescent dissecting microscope. Embryos, or fish into which they grow, may be screened for the presence of a defect resulting from the
20 transgene. In another approach, embryos may be pooled prior to extraction of genomic DNA and analysis of the genomic DNA by PCR and/or restriction enzyme digest. Positive clones may be expanded and developed into breeding fish. These fish may then be bred to produce fish which carry one copy of the gene
25 construct in the germ line. These heterozygous fish may then be bred to produce fish carrying the gene homozygously.

A further aspect provides a method which includes introducing a nucleic acid construct wherein a coding sequence of a desired
30 disease gene is placed under control of an eye-specific promoter into a fish, e.g. zebrafish, embryo cell. DNA may be injected

directly *in vivo* into cells of an early embryo. With the
establishment of embryonic stem cell culture, other methods
generally referred to without limitation as "transformation", may
be employed, for instance selected from any method available in
the art, such as using calcium phosphate transfection, DEAE-
Dextran, electroporation, liposome-mediated transfection and
transduction using retrovirus or other virus. Marker genes such
as antibiotic resistance or sensitivity genes may be used in
identifying clones containing nucleic acid of interest, as is
well known in the art.

In one particular embodiment, a coding sequence of a disease gene
to be introduced into the fish, e.g. zebrafish, is placed under
regulatory control of the ath5 promoter. Ath5 is the zebrafish
eye-specific ortholog of the Drosophila basic helix-loop-helix
transcription factor Atonal and is expressed specifically in
retinal ganglion cells (Kay et al. Neuron. 2001 Jun;30(3):725-
36). In another embodiment, the promoter of the disease gene is
replaced with the opsin promoter (Kennedy et al., (2001) J. Biol.
Chem. 276(17): 14037-43) or rhodopsin promoter.

In a further aspect, the present invention provides a method of
making a fish, such as a zebrafish, useful in or for use in a
screen as disclosed herein and discussed further below. Such a
method may comprise providing a gene construct wherein a coding
sequence of a disease gene is operably linked to a promoter that
is an eye-specific promoter in the fish, e.g. zebrafish,
introducing the gene construct into a fish, e.g. zebrafish,
embryo, causing or allowing the gene construct to integrate into
the fish embryo genome, and growing the fish embryo into a viable
fish.

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A viable and reproductive fish, e.g. zebrafish, may mate with one or more other fish, establishing a line of fish, e.g. zebrafish, transgenic for the gene construct comprising the disease gene operably linked to, and under regulatory control of, the eye-specific promoter. A line of such fish, e.g. zebrafish, is useful in suppressor gene screens as disclosed.

In order to introduce a disease gene into a fish embryo, e.g a zebrafish embryo, a gene construct is made, using techniques available to those skilled in the art. The construct may be released from a vector by restriction digest, and gel purified, for example by elution in 1xTE (pH8.0) and dilution to a working concentration of 50-100 ug/ml KCl containing a marker dye such as tetramethyl-rhodamine dextran (0.125%). Typically, 1 to 3 nl of this solution may be injected into single celled zebrafish embryos. Several thousand embryos may be injected.

Injected embryos are grown up and then mated with each other or to a non-transgenic wild-type fish. Transmission of the transgene to the subsequent generation is usually mosaic, ranging from 2 to 90%. At least 100 offspring are typically analysed to establish whether the founder fish carriers the transgene.

The injected embryos may be reared and assessed for visual function at intervals (e.g. 2 days) by means of modified optokinetic and optomotor assays, e.g. as discussed further below.

Fish demonstrating visual impairment may be grown up and may be mated with wild-type fish. The parents and offspring may be

17

matched and the offspring similarly assessed for visual
dysfunction. Those offspring with visual dysfunction, and hence
likely germline transmission of an integrated disease gene
construct, can be selectively bred. Some of the offspring may be
5   sacrificed for more detailed analysis, e.g. to confirm the nature
of the blindness. This analysis may include *in situ* hybridisation
studies using sense and anti-sense probes to the introduced gene
to check for expression of the construct in the ganglion cell
layer, anatomical assessment such as with plastic sections to
10   check for the degeneration of the ganglion cell layer; and
terminal deoxyuridine nucleotide end labelling (TUNEL) to check
for apoptotic cell death in the ganglion cell layer.

Families from which fish with the appropriate characteristics
15   came may be maintained through subsequent generations. This
maintenance then allows this new mutant strain to be entered into
a secondary suppressor screen in accordance with further aspects
of the invention.

20   Another aspect of the present invention provides cells of
transgenic fish, such as zebrafish, fugu, goldfish, medaka or
giant rerio as disclosed, whether isolated cells or cell lines
derived from the fish and optionally immortalised using standard
techniques.

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A gene such as a disease gene sequence (e.g. heterologous to
fish, such as heterologous to zebrafish) to be employed in
aspects and embodiments of the present invention may employ a
wild-type gene or a mutant, variant or derivative sequence may be
30   employed. The sequence may differ from wild-type by a change
which is one or more of addition, insertion, deletion and

18

substitution of one or more nucleotides of the sequence shown.
Changes to a nucleotide sequence may result in an amino acid
change at the protein level, or not, as determined by the genetic
code.

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It is well known that pharmaceutical research leading to the
identification of a new drug may involve the screening of very
large numbers of candidate substances, both before and even after
a lead compound has been found. This is one factor which makes

10   pharmaceutical research very expensive and time-consuming. Means
for assisting in the screening process can have considerable
commercial importance and utility. Such means for screening for
substances potentially useful in treating or preventing a
disorder or disease is provided by fish such as zebrafish

15   according to the present invention. Suppressor genes identified
using the invention and substances that affect activity of such
suppressor genes represent an advance in the fight against
disease since they provide basis for design and investigation of
therapeutics for *in vivo* use, as do test substances able to

20   affect activity or effect of a treatment, and substances that
affect activity or effect of expression of a disease gene in a
fish.

In various further aspects the present invention relates to
25   screening and assay methods and means, and substances identified
thereby.

The present inventor has realised that fish such as zebrafish are
useful in a secondary suppressor screen. A secondary suppressor
30   screen involves introducing one or more mutations into the genome

19

and screening or selecting for negation or suppression of the
effect of a primary mutation.

The principle can be illustrated by way of example, with
reference to a hypothetical gene B of which normal function is to
control fish size. If this gene is mutated so that it is
overactive, bigger fish will ensue. Now take another hypothetical
gene S whose normal function is to make cells smaller. If this
gene is mutated the cells will be bigger. Thus if a mutation is
introduced into the S gene in a cell already harbouring a mutated
B gene, the two will cancel each other out and the cell will be
normal sized. The mutated S gene suppresses the phenotype of the
mutated B gene.

However, there is a problem where gene B is a dominant disease
gene, since if this makes the fish non-viable, causes them to die
quickly or fail to reproduce, it will not be possible to raise
adult fish harbouring a mutation in gene B.

The present invention provides a solution to this problem by
restricting expression of the disease gene to the eye, by placing
it under the control of an eye-specific promoter. As a result of
this restricted expression, the disease process is limited to
these specific cells. The fish are viable, can be raised to
adulthood and bred, and are thus amenable to use in a secondary
suppressor screen.

Furthermore, the invention provides for an accurate, gradable and
rapid screening method for the presence of disease activity and
degree of disease activity. Degeneration of the retina results
in blindness and it is straightforward to assay for blindness and

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impairment of visual function in fish such as zebrafish in
accordance with the present invention.

The fish, e.g. zebrafish, provided by the present invention are
5   useful in screens for suppressor genes that reduce activity or
effect of a disease gene. According to a further aspect of the
present invention there is provided the use of a fish, e.g.
zebrafish, fugu, goldfish, medaka or giant rerio in such a
screen.

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In a further aspect, the present invention provides a method of
screening for a suppressor gene that lessens activity or effect
of a disease gene, the method comprising:
      providing fish, e.g. zebrafish transgenic for a disease gene
15 under regulatory control of an eye-specific promoter, wherein
expression of the disease gene within the eye of the fish affects
vision of the fish, as model fish for screening;
      subjecting said model fish to mutation to provide mutated
fish;
20   comparing vision of mutated fish with vision of model fish
in order to identify any mutated fish with altered vision
compared with model fish;
      identifying a genetic difference between model fish and any
mutated fish with such altered vision, thereby to identify a
25 suppressor gene that lessens activity or effect of the disease
gene.

As noted, preferred embodiments of the present invention in its
various aspects employ zebrafish.

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A fish such as a zebrafish harbouring a disease-causing or
phenotype-altering mutation manifested by visual impairment as a
result of expression of the disease gene in the eye may be
identified on the basis that it does not display the visual
5   impairment or does not display it to the same degree because of
the effect of a mutation in a second gene, this mutated second
gene suppressing the activity of the disease gene.

Visual function of a fish such as a zebrafish may for example be
10   assayed one of the following ways:

(1)   Zebrafish change colour to blend in with background. In a
dark environment the fish turn blacker and in a bright
environment lighter. Blind fish perceive themselves as being in
15   the dark and thus turn black. This provides a very rapid visual
screen - the black fish are blind (Neuhauss).

(2)   Zebrafish or other fish optokinetic response can be assayed
by passing stripes in front of the eyes. As in human subjects,
20   the resultant reflexive eye movements cannot be suppressed. Thus
passage of stripes in front of fish such as zebrafish and the
assessment of the presence or absence of optokinetic response
provides another assay for visual function. A related visual
assay involves the optomotor response. The sophistication of
25   stimuli may be built up to allow a detailed, graded assessment of
fish, e.g. zebrafish, visual function (Orger). Moreover, the
assessment mechanisms allow for the testing of larger numbers of
fish in a short period of time.

30   A moving grating or a movie, e.g. presented as a computer-
animated display on a screen, elicits innate optomotor behavior

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in zebrafish larvae; they swim in the direction of perceived
motion (Orger et al. Nat Neurosci 2000 Nov 3(11):1128-33).
Zebrafish larvae innately begin responding to moving stimuli
shortly after hatching. This is advantageous in a screen of the
5   present invention as disclosed herein, since it allows for rapid
determination of the effect of mutation on a model zebrafish.
Other fish show similar responses.

Visual function of zebrafish may be determined by considering
10   their colour or background pigmentation, alteration of which is
indicative of blindness as discussed. Visual function may be
determined by means of observation of response to a pattern of
e.g. of stripes passed in front of the eyes of the fish. In a
preferred embodiment, black fish expected to be blind and
15   lighter-coloured fish expected to have visual function are
segregated on the basis of their colour, prior to determination
of the degree or extent of visual function by means of a visual
assay.

20   Assaying for visual function of a fish such as a zebrafish may
employ observation of a change in background pigmentation, of
visual startle, of optokinetic response and/or optomotor
response. The latter two both typically involve passage of a
pattern, e.g. horizontal stripes, passed. In an optokinetic
25   assay, fish may be immobilized in 3% methylcellulose and the
pattern passed in front of the eyes. In an optomotor assay, fish
are free swimming in one or more long, narrow channels, and the
pattern is passed along the length of the channels. Fish swim in
the perceived direction of motion of the stipes and thus collect
30   at one end of the channel, unless they are blind in which case
they are distributed randomly.

23

A typical powerful stimulus is a 100% contrast square wave
filling the entire visual field, and a stripe width of 36 degrees
moving at 4 Hz. Limits of vision are identified in Orger et al.,
5   supra, although for partially blind fish the limits are less.
Degree of visual dysfunction can be determined by decreasing the
contrast and/or stripe width of a pattern until the fish no
longer respond. Other aspects of visual dysfunction may be
analysed by varying the colour of the stripes or other pattern.
10

The pattern may be stripes or wavy lines moving across the field
of vision. Other non-Fourier or second order stimuli may be
employed, e.g. as described in Orger et al., supra.

15   Thus, in embodiments of the invention fish such as zebrafish
transgenic for a disease gene expressed in the fish under control
of an eye-specific promoter may be maintained through several
generations. This maintenance allows this strain to be entered
into a secondary suppressor screen, through which a secondary
20   mutation is introduced at random into the genome of this strain.
The visual function of fish now harbouring both the disease
causing mutation and the potentially suppressing secondary
mutation may be assessed by means of a modified optomotor or
optokinetic assay. Fish in which the visual function is better
25   than the initial subjects of the mutation are candidates for
harbouring a second mutation which is suppressing the effect of
the primary disease causing mutation. This second mutated gene
and its protein product are therapeutic targets for treatment of
the disease caused by the primary mutation.
30

A mutant fish such as a mutant zebrafish transgenic for a disease
gene under control of an eye-specific promoter and containing a
mutation within a suppressor gene that lessens activity or effect
of the disease gene on visual function of the fish is itself
useful in a further assay for a test substance able to modulate
or affect, preferably potentiate or increase the suppression
effect of the suppressor gene. Clearly, the same applies where a
mutation in a gene is identified that enhances or increases
activity of a second gene.

Of course, the person skilled in the art will design any
appropriate control experiments with which to compare results
obtained in test assays.

A number of strategies are available to the ordinary skilled
person for introducing a secondary mutation into a primary mutant
strain. Such strategies include breeding female fish carrying
the transgene to homozygosity and then crossing these to F1 or F2
generation males, derived from F0 males exposed to
ethylnitrosourea (ENU). The mutagenesis procedure as described by
van Eeden et al (Methods Cell Biol 1999 60) typically yields
mutation rates of $0.9-3.3 \times 10^{-3}$ per loci. A starting number of 100
healthy fertile males are considered necessary to obtain 20-30
fertile males after 6 treatments with 3mM ENU.

Mutagenesis may be performed as follows:

Ethylnitrosourea (ENU) is dissolved in acetic acid to a final
concentration of 10mM, as determined by the optical density at
238nm at pH6.0 (extinction coefficient = 5830/M/cm), and then
diluted to a working concentration of 3.0mM in 10mM sodium

25

phosphate buffer, pH 6.6. Males which reliably produce fertilised offspring are placed in ENU solution for 1 hour. After the procedure the fish are washed in 2 changes of aquarium water for 1 hour each time, prior to return to the aquarium. The

5   mutagenesis procedure is repeated up to 6 times at weekly intervals.

The frequency of mutations induced is proportional to the exact number of mutagenesis procedures performed. The number of

10   procedures can thus be varied depending on the number of mutations desired per genome.

The actual mutagenesis procedure is best carried out in the dark to minimise the stress to the fish.

15

Initial progeny from the mutagenised fish are mosaic. The mutagenzed fish are therefore mated 3 times at weekly intervals following the final mutagenesis procedure. Progeny obtained after this will be non-mosaic, since any mutations will have arisen in

20   spermatogonial stem cells.

Other useful mutagenesis agents include gamma- or X-ray-mediated mutagenesis, and retrovirus-mediated insertional mutagenesis.

25   An alternative method to overcome the problems of lethality and thus allow dominantly acting disease mutations to be entered into a secondary suppressor screen involves placing the mutated gene under the control of an inducible promoter, such as a heat shock promoter, the tetracycline inducible system (Clontech) or a

30   hormonal inducible promoter.

26

The tetracycline inducible system (Clontech) relies on two
different constructs. The tet-on plasmid expresses the
tetracycline controlled transactivator, rtTA, consisting of a
mutated tet-repressor protein, rTetR, coupled to the VP16
5   activation domain of the herpes simplex virus (AD). The rtTA
protein binds to and activates the tetracycline response element
(TRE) part of the second plasmid. The activated TRE acts with the
CMV silent promoter to drive expression of the gene of interest.
The key to the system is that the rtTA transactivator will only
10   bind the TRE response element in the presence of tetracycline (or
its analogue doxycycline). In the absence of tetracycline,
binding does not take place, the response element is "off" and
protein expression is minimal, such that even toxic proteins can
be effectively switched on and off (Harkin et al Cell 1999
15   97:575-86; Lee et al PNAS 1988 95:11371-6).

The tetracycline inducible system has been used in mammals to
provide regulated overexpression of interleukin 11 in the lungs
of mice (Ray et al 1997 J Cline Inv 100: 2501-). There was no
20   apparent toxicity to embryos treated with doxycycline in utero,
or to pups or adults. In the absence of doxycycline levels of IL-
11 were less than 50pg/ml. Doxycycline induction raised levels to
greater than 0.3ng/ml. On removal of doxycycline from the
drinking water IL-11 levels fell by >80% within 24h. Alterations
25   to the response element are possible, giving even tighter
control.

A further modification involves the coupling of the TRE response
element to a bi-directional promoter. This allows eGFP to be
30   expressed whenever the target gene is also expressed. This
provides an easy visual marker of gene carriage and activation.

Thus in the scenario of a secondary suppressor screen, a mutated
gene is introduced into the fish, e.g. zebrafish, under the
control of an inducible system (e.g. tetracycline inducible). The
gene is switched on by addition of the inducer (e.g.
tetracycline) and those fish expressing the gene selected for
further study. The gene is then switched off, allowing the fish
to be reared through subsequent generations and thus allowing
additional mutations to be introduced as part of a secondary
suppressor screen.

Accordingly, in a further aspect, the invention provides a method
of screening for a suppressor gene that lessens activity or
effect of a disease gene, the method comprising:
    providing fish such as zebrafish as model fish for
screening, which model fish are transgenic for a gene construct
wherein a coding sequence a for disease gene that is lethal in
fish or renders fish non-viable or non-reproductive is provided
under control of an inducible promoter and the model fish are
generated and reared under conditions in which the inducible
promoter is not induced and/or is repressed;
    subjecting the model fish to mutation to provide mutated
fish and inducing and/or de-repressing the inducible promoter to
cause expression of the disease gene in the model fish;
    determining viability and/or reproductivity of mutated fish
in which the disease gene is expressed in comparison with model
fish in which the disease gene is expressed;
    identifying a genetic difference between model fish and any
mutated fish with altered viability and/or reproductivity,
thereby to identify a suppressor gene that lessens activity or
effect of the disease gene.

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The suppressed strain of fish will have at least two mutations:
the disease-causing/phenotype-inducing mutation resulting from
introduction of the disease gene, and the disease-/phenotype-
5   suppressing or repressing mutation.

It should be noted also that in place of identification of a
suppressor mutation and gene, the present invention in any of its
aspects and embodiments may be used to identify a mutation and
10   gene that enhances or increases the severity of the primary
phenotype. This can be used to identify additional genes
involved in a particular disease pathway.

Similarly, the present invention may be used to identify a test
15   substance that affects activity or effect of a gene in a fish or
a treatment of a fish, e.g. where the gene is a transgene
expressed in the eye and the transgene or the treatment has an
effect on vision of the fish.

20   A screening or assay method according to an aspect or embodiment
of the present invention may comprise identifying a suppressor
gene that lessens activity or effect of a disease gene.

A screening or assay method according to an aspect or embodiment
25   of the present invention may comprise identifying a gene that
enhances or increase activity or effect of a second gene, or a
test substance that affects activity or effect of the second
gene, whether lessening or decreasing, or enhancing or increasing
such activity or effect.

30

Following identification of a gene which affects activity or effect of a second gene, e.g. a suppressor gene, the gene (including a homologue in another species, e.g. human) or encoded gene product may be cloned or otherwise provided in an isolated

5   or purified form, and may be provided in a composition comprising at least one additional component.

The gene, e.g. suppressor gene, (including a homologue in another species, e.g. human) or a gene product encoded by the gene, e.g.

10   suppressor gene, may be used in a screening system for assaying ability of a test substance to affect activity of the gene or the gene product encoded by the gene.

A test substance that affects activity of a gene, e.g. a

15   suppressor gene, or the gene product encoded by the gene may be provided in a composition comprising at least one additional component.

Following identification of a suppressor gene for a disease gene

20   of interest, or other gene that affects activity or effect of a second gene, the suppressor or other gene and/or an encoded gene product may be employed as a target for identification of potential therapeutics or as a therapeutic in its own right. Also, the nature of the suppressing or other effect may be

25   investigated further.

The suppressor or other gene that affects activity or effect of a second gene may be a novel gene or may be a known gene not previously known to have a function of affecting or suppressing

30   activity or effect of the relevant disease gene. The gene may be one already known or suspected to have function in affecting or

suppressing activity, in which case the results from the fish
assay add weight to the available evidence. In particular, the
fact that the suppression or other effect occurs *in vivo*
increases the confidence for using the gene, or encoded gene
5 product or fragment thereof, or a component in the pathway of
action of the gene or gene product, as a drug target. For
further investigation and use, a homologue from another species
may be used, where available e.g. via use of cloning or screening
technology.

10

The responsible mutation, e.g. suppressive mutation, may be
identified by using mapping techniques available in the art,
(e.g. see Detrich H.W., Zon L.I. & Westerfield M. (1998) The
Zebrafish: Genetics and Genomics. Methods in Cell Biology.
15 Volume 60, pg 182-192).

Thus, to identify the position of the relevant mutation, e.g. a
suppressive mutation, the mutant locus is mapped relative to the
position of a marker, the position of which is known. DNA markers
20 include short sequences of DNA, cloned genes or other mutations.
The current best method in zebrafish involves simple sequence
length polymorphisms (SSLPs) as they cover the entire genome at
high density. It is therefore possible to map to within 0.5cM,
from which either a chromosomal walk may be initiated, further
25 mapping may be undertaken using single strand conformational
polymorphisms, or candidate genes selected directly.

Mapping using SSLP

30 These markers consist of 2 primers flanking a dinucleotide (CA)
repeat. These are extremely variable in length & polymorphic

31

between zebrafish strains. The SSLP mapping involves the
following steps:

1. Raising a map cross, identifying mutant carriers, fixing
   mutant & sibling progeny separately
2. Isolating genomic DNA from both mutants & siblings
3. Genome scanning using pooled DNA from both mutants &
   siblings to determine linkage group
4. Verifying potential linkages with single embryo DNA
5. Searching for closely linked markers
6. Positioning the mutation on the genetic map by determining
   the number of recombinations between marker & mutation.

Isolation of genomic DNA

To extract DNA from single embryos, embryos fixed in 100%
methanol are poured into a petri dish. More methanol is added to
the dish to ensure the embryos remain covered. Embryos are then
pipetted into a 96 well plate: a single embryo per well. A
pipette is then used to remove as much methanol as possible from
around the embryos. The remaining methanol is then evaporated off
on a PCR block set at 70°C for 15 minutes. 25ul of a mix of 250ul
proteinase K (17mg/ml, Merck) & 2.25ml 1xTE, is added to each
well. The PCR plate is then covered with Hybaid film & heated in
a PCR machine for 240 minutes at 55°C, followed by a 10 minute
75°C incubation to inactivate the proteinase K. The plates can be
kept at -20°C until needed.

Genome scanning

Pooled DNA is prepared by taking 10ul from each of 48 single
samples, and then diluted to a final concentration of 50ng/ul.
Primers for markers are arranged on a master primer 96 well plate

in such a way that the mutant & sibling sample analysed with the
same marker will subsequently run adjacent to each other on an
agarose gel. The markers selected for the PCR plates are those
known to show useful polymorphisms & which evenly span the entire
5   genome.

PCR reactions are then set up in 96 well format. Each well
contains 14.28ul PCR mix, 0.16ul each of 20uM forward & reverse
primer, 0.4ul of 5U/ul Taq polymerase & 5.0ul of template DNA.
10   PCR is performed with initial denaturing at 94°C for 3 minutes,
followed by 35 cycles of denaturing at 94°C for 30 seconds,
annealing at 60°C for 30 seconds & primary extension at 72°C for
1 minute. The reaction is completed by a final 5 minute extension
at 72°C.

15

PCR Mix
0.2mM dATP
0.2mM dCTP
0.2mM dGTP
20   0.2mM dTTP

in PCR buffer

PCR buffer (10x)
25   100mM Tris-HCl, pH 8.3
500mM KCl
15mM $MgCl_2$
0.1% (w/v) gelatin

33

Single-embryo PCR

PCR reactions are set up and performed as above, except that
single embryo DNA is used as the template.

5   The PCR products are assessed for polymorphisms by running out on
a 2% agarose gel at 200V for 80 minutes in 1x TBE.

Mapping using SSCP

10   This uses single strand DNA. Each strand assumes its
thermodynamically preferred conformation. Single nucleotide
substitutions may alter the conformation sufficiently for a
difference in migration pattern to be detected on a non-
denaturing gel. This allows non-SSLP markers tightly linked to
15   the mutation to be analysed.

The protocol used to amplify a marker is as for SSLP mapping. To
precipitate the PCR products, 2 volumes of pre-cooled 100%
ethanol and 0.1 volume of 3M Na-acetate are added to the PCR
20   product, vortexed well, incubated for at least 20 minutes at –
20°C & centrifuged in a cooled centrifuge at 13000 rpm for 25
minutes. The supernatant is discarded, the DNA pellet air-dried &
resuspended in 8ul of ddH_2O. To 5.4ul of PCR product, 0.6ul of
denaturing solution & 2.4ul of loading buffer are added & briefly
25   mixed, prior to incubation at 85°C for 10 minutes. The sample is
then quickly chilled on ice. 6-8ul of each sample is then loaded
onto a native precast acrylamide gel (CleanGel SSCP, ETC
Elektrophorese-Technik) & run at 200V & 15°C following the
manufacturer's instructions.

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Denaturing solution
10mM EDTA
500mM NaOH

5   *Loading buffer*
2% bromophenol blue
2% xylenecyanol
in formamide

10 The gels are then stained using a silver staining kit (PlusOne
DNA Silver Staining Kit, Pharmacia), as per the manufacturer's
instructions.

By these methods the mutation is mapped close enough to select a
15 candidate gene. This gene is then sequenced in both mutant wild-
type fish to identify mutations.

If the suppressor or other gene that affects activity or effect
of a second gene encodes a protein, it may be that that protein
20 interacts with or binds the second gene, e.g. disease gene, or
gene product. Thus, for example, a novel protein-protein binding
pair may be identified, immediately presenting the possibility of
modulating or affecting such binding as a target for identifying
candidate therapeutics.
25

Where interaction or binding between gene products is to be
investigated further or employed in assay methods for identifying
further substances able to affect the binding or interaction,
suitable approaches are available in the art, for instance
30 techniques involving radioimmunoassay, co-immunoprecipitation,
scintillation proximity assay, ELISA methods, and two-hybrid

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assays (see e.g. Fields and Song, 1989, Nature 340; 245-246), for instance using the two binding domains of the GAL4 transcription factor or the LexA/VP60 system.

5   Further mutation in the suppressor or other gene may be used to identify variants with enhanced or otherwise altered suppressor function.

Thus, the suppressor gene or other gene, or encoded gene product,
10   in wild-type or a mutated form (which may be a mutated form as identified in the original screen or a further mutated form) may be used in a therapeutic composition.

In various further aspects, the present invention thus provides a
15   pharmaceutical composition, medicament, drug or other composition comprising a suppressor gene or other gene or gene product or substance found to affect the disease gene of interest or suppression of the disease gene of interest, the use of such a material in a method of medical treatment, a method comprising
20   administration of such a material to a patient, e.g. for treatment (which may include preventative treatment) of a medical condition, use of such a material in the manufacture of a composition, medicament or drug for administration for such a purpose, e.g. for treatment of a proliferative disorder, and a
25   method of making a pharmaceutical composition comprising admixing such a material with a pharmaceutically acceptable excipient, vehicle or carrier, and optionally other ingredients.

Whatever the material used in a method of medical treatment of
30   the present invention, administration is preferably in a "prophylactically effective amount" or a "therapeutically

36

effective amount" (as the case may be, although prophylaxis may
be considered therapy), this being sufficient to show benefit to
the individual. The actual amount administered, and rate and
time-course of administration, will depend on the nature and
5   severity of what is being treated. Prescription of treatment,
e.g. decisions on dosage etc, is within the responsibility of
general practitioners and other medical doctors.

Pharmaceutical compositions according to the present invention,
10   and for use in accordance with the present invention, may
include, in addition to active ingredient, a pharmaceutically
acceptable excipient, carrier, buffer, stabiliser or other
materials well known to those skilled in the art. Such materials
should be non-toxic and should not interfere with the efficacy of
15   the active ingredient. The precise nature of the carrier or
other material will depend on the route of administration, which
may be oral, or by injection, e.g. cutaneous, subcutaneous or
intravenous.

20   Pharmaceutical compositions for oral administration may be in
tablet, capsule, powder or liquid form. A tablet may include a
solid carrier such as gelatin or an adjuvant. Liquid
pharmaceutical compositions generally include a liquid carrier
such as water, petroleum, animal or vegetable oils, mineral oil
25   or synthetic oil. Physiological saline solution, dextrose or
other saccharide solution or glycols such as ethylene glycol,
propylene glycol or polyethylene glycol may be included.

For intravenous, cutaneous or subcutaneous injection, or
30   injection at the site of affliction, the active ingredient will
be in the form of a parenterally acceptable aqueous solution

which is pyrogen-free and has suitable pH, isotonicity and
stability. Those of relevant skill in the art are well able to
prepare suitable solutions using, for example, isotonic vehicles
such as Sodium Chloride Injection, Ringer's Injection, Lactated
5   Ringer's Injection. Preservatives, stabilisers, buffers,
antioxidants and/or other additives may be included, as required.

Examples of techniques and protocols mentioned above can be found
in Remington's Pharmaceutical Sciences, 16th edition, Osol, A.
10   (ed), 1980.

Vectors such as viral vectors have been used in the prior art to
introduce nucleic acid into a wide variety of different target
cells. Typically the vectors are exposed to the target cells so
15   that transfection can take place in a sufficient proportion of
the cells to provide a useful therapeutic or prophylactic effect
from the expression of the desired peptide. The transfected
nucleic acid may be permanently incorporated into the genome of
each of the targeted cells, providing long lasting effect, or
20   alternatively the treatment may have to be repeated periodically.

A variety of vectors, both viral vectors and plasmid vectors, are
known in the art, see US Patent No. 5,252,479 and WO 93/07282.
In particular, a number of viruses have been used as gene
25   transfer vectors, including papovaviruses, such as SV40, vaccinia
virus, herpesviruses, including HSV and EBV, and retroviruses.
Many gene therapy protocols in the prior art have used disabled
murine retroviruses.

30   As an alternative to the use of viral vectors in gene therapy
other known methods of introducing nucleic acid into cells

38

includes mechanical techniques such as microinjection, transfer
mediated by liposomes and receptor-mediated DNA transfer, also
administration of naked DNA or RNA, by simple administration,
e.g. injection, of nucleic acid such as a plasmid, for instance
5   to muscle.

Specific disease models which take advantage of such an approach
are described. These specific examples are offered by way of
illustration and not by way of limitation.
10

All documents mentioned anywhere in this specification are
incorporated by reference.

EXAMPLE 1 - Parkinson's disease
15

Parkinson's Disease affects 1 in 100 of the population. 0.5% of
multiplex families with Parkinson's Disease harbour a pathogenic
mutation in the alpha-synuclein gene (Polymeropolous et al 1996).
Whilst it does not appear to be a major risk factor for familial
20   or non-familial Parkinson's Disease it provides a useful general
model of Parkinson's Disease pathogenesis. Neuronal
overexpression of wildtype asynuclein resulted in progressive
accumulation of inclusions in the neocortex, hippocampus and
substantia nigra associated with loss of dopaminergic terminals
25   and motor impairment (Masliah et al 2000) and alpha-synuclein is
present in the Lewy Bodies of patients with sporadic Parkinson's
Disease (Spillantini et al Nature 1997) leading to the conclusion
that accumulation of wild type alphasynuclein may be involved in
the pathogenesis of Parkinson's Disease. Indeed, the concept of
30   synucleinopathies is emerging as a term to embrace both
Parkinson's Disease, Multiple System Atrophy (MSA) and Lewy Body

39

Dementia (LBD). (MSA is the underlying diagnosis in about 10 % of
parkinsonian patients; Lewy Body dementia is becoming recognised
as the commonest cause of dementia after Alzheimer's Disease).

5   In an embodiment of the present invention, a mutant alpha-
synuclein encoding sequence is ligated to an eye specific
promoter and the resulting construct is introduced into the
zebrafish genome. In a particular example, mutant human alpha-
synuclein is ligated to the zebrafish ath5 promoter. The contruct
10   is introduced into zebrafish embryos and the vision of the
resulting fish assayed, to identify fish in which alpha-synuclein
is expressed in the eye. These fish are then selected for use in
a secondary suppressor screen.

15   *EXAMPLE 2 - Huntington's Disease*

Huntington's Disease is an incurable, dementing condition which
affects 4-7/100,000 of population. Genetic testing means affected
patients are often identified long before they become
20   symptomatic, but there is still no treatment.

Huntington's disease is due to an expanded CAG repeat sequence in
the huntingtin gene with a resultant expanded polyglutamine chain
in the protein. A similar mechanism underlies the other triplet
25   repeat diseases described to date (SCA1-8, Kennedy's disease or
X-linked bulbo-spinal neuronopathy and DRPLA or
dentatorubropallidoluysian atrophy). The pathogenesis of all the
triplet repeat diseases is believed to be similar. Indeed
expanded polyglutamine peptides on their own result in cell death
30   and neurodegeneration (Marsh et al HMG 2000 9 13-25). It seems
that the remainder of the protein modifies the intrinsic toxicity

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of the polyglutamine repeat, rendering particular cell groups
either more resistant or sensitive (Marsh et al 2000).

In an embodiment of the present invention a zebrafish model of
5   Huntington's Disease amenable for use in a secondary suppressor
screen is generated by ligation of mutant human or zebrafish
huntingtin to a zebrafish eye specific promoter, such as the ath5
promoter, and subsequent creation of an ath5-promoter-mutant
huntingtin zebrafish line. These fish are then useful in a
10   secondary supressor screen to identify genes which alleviate
Huntington's Disease.

EXAMPLE 3 - Spinal muscular atrophy

15   Spinal muscular atrophy (SMA) is the commonest autosomal
recessive disease after cystic fibrosis with an incidence of 1 in
6000 births. It is due to degeneration of the anterior horn cells
in the spinal cord with consequent wasting and weakness of all
muscles. The rate and severity of muscle wasting varies with
20   milder cases surviving into adulthood. Unfortunately, most
commonly death occurs from respiratory failure in early
childhood. There are no treatments for SMA available, and none in
clinical trial.

25   Although the pathology is localised to the anterior horn cells,
the causative gene, SMN1 or survival of motor neuron, is widely
expressed throughout the body. The SMN protein is involved in RNA
splicing, partly through its participation in small nuclear
ribonucleoprotein (snRNP) assembly in the cytoplasm (Pellizzoni
30   et al Cell 1999 96 1167). The paradox which is challenging SMN
researchers is why a protein involved in a process apparently

essential to all cells should, in its mutant form, only be deleterious to anterior horn cells.

An embodiment of the present invention comprises creating a
5   zebrafish model of SMA amenable for use in a secondary suppressor
screen by ligation of human SMN to a zebrafish eye specific
promoter, such as the ath5 promoter, and subsequent generation of
an ath5-promoter-mutant SMN zebrafish line. These fish are
useful in a secondary supressor screen to identify genes which
10   alleviate SMA. Overexpression of the wild type human SMN in flies
is known to result in an SMN phenotype through a dominant
negative phenotype (Miguel-Aliaga et al FEBS Letters 2000 486
99-102).

15   *EXAMPLE 4 - Motor neuron disease*

Motor neuron disease (MND) is a devastating neurodegeneration in
which progressive dying away of any combination of the motor
neurons, anterior horn cells or corticospinal tract results in
20   progressive wasting away of all of the muscles over several
years, culminating in a wheelchair-bound patient unable to speak,
swallow and eventually breathe. It affects 1 in 1000 of the
population. 10% of cases are familial and 20% of these are due to
mutations in the Cu/Zn-superoxide dismutase (SOD-1) gene (Rosen
25   et al 1993). The role of SOD-1 in free radical scavenging
suggested that excitotoxic mechanisms could be important in MND
pathogenesis. This has been supported by findings in both motor
neuron cultures and studies in non-SOD-1 MND patients (e.g.
Rothstein et al 1992 and 5; Aoki et al 1998; Rothstein and Kuncl
30   1995). In drosophila, SOD1 mutations shorten the life span of the
fly, whilst overexpression of normal human SOD-1 rescues the

42

mutant phenotype and also extends the life span of wildtype
flies, even if the human SOD-1 is only overexpressed in motor
neurons (Parkes et al Nat Gen 1998 19 171-4).

5   A further embodiment of the present provides for creation of a
      zebrafish model of MND amenable for use in a secondary suppressor
      screen, involving ligation of mutant human or zebrafish SOD-1 to
      a zebrafish eye specific promoter, such as the ath5 promoter, and
      subsequent creation of an ath5-promoter-mutant huntingtin
10   zebrafish line. A secondary suppressor screen may then employ
      such fish to identify genes which alleviate MND.

      EXAMPLE 5 - Multiple Sclerosis

15   This chronic disabling condition affects 1 in 100 of the
      population. Current immunomodulatory therapies such as betaIFN
      have only weak efficacy in a subset of sufferers. Moreover, they
      need to be given subcutaneously and frequently have side-effects.

20   Overexpression of TNF alpha has been validated as an experimental
      model system in mice (Probert et al. (1995) Proc. Natl. Acad.
      Sci. USA 92(24): 11294-8). A zebrafish model of MS amenable for
      use in a secondary suppressor screen may be generated as an
      embodiment of the present invention by ligation of a TNFalpha
25   gene to a zebrafish eye specific promoter, such as the ath5
      promoter, and subsequent creation of an ath5-promoter- TNFalpha
      zebrafish line. Putting these fish into a secondary supressor
      screen allows for identifying genes which alleviate MS. This
      model is particularly useful because the ganglion cells are
30   unique in the retina, in that their axons give rise to the optic
      nerve, a common site of disease in MS. Such a model would not

43

function if the TNFalpha gene was overexpressed in any other
retinal cell type. It is the peculiar property of the ganglion
cells giving rise to a central nervous system tract of myelinated
tissue which makes this possible. The onset of disease in
5   zebrafish would therefore result in demyelination of the optic
nerve, hence blindness and an easily scoreable MS phenotype.

44

CLAIMS:

1.   A method of screening for a substance or gene (termed herein
"first gene") that affects activity or effect of a second gene,
5   or activity or effect of a treatment, on a fish, the method
comprising:

providing, as model fish for screening, (i) fish transgenic
for the second gene, wherein the second gene is under regulatory
control of an eye-specific promoter and expression of the second
10   gene within the eye of the fish affects vision of the fish, or
(ii) fish subject to said treatment, wherein the treatment
affects vision of the fish;

mutating said model fish to provide mutated fish or treating
said model fish with a test substance to provide treated fish;

15   comparing vision of mutated fish or treated fish with vision
of model fish in order to identify any mutated fish or treated
fish with altered vision compared with model fish;

thereby to identify a test substance that affects activity
or effect of the second gene or activity or effect of said
20   treatment, or, by identifying a genetic difference between model
fish and mutated fish with such altered vision, to identify a
first gene that affects activity or effect of the second gene or
activity or effect of said treatment.

25   2.   A method according to claim 1 comprising mutating model fish
transgenic for the second gene to provide mutated fish and
identifying a first gene that affects activity or effect of the
second gene.

30   3.   A method according to claim 1 comprising treating with a
test substance model fish transgenic for the second gene to

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provide treated fish and identifying a test substance that
affects activity or effect of the second gene.

4.   A method according to claim 1 comprising mutating model fish
5   subject to said treatment to provide mutated fish and identifying
a first gene that affects activity or effect of said treatment.

5.   A method according to claim 1 comprising treating with a
test substance model fish subject to said treatment to provide
10   treated fish and identifying a test substance that affects
activity or effect of said treatment.

6.   A method according to claim 1 comprising identifying a first
gene that lessens activity or effect of the second gene.
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7.   A method according to claim 6 wherein the second gene is a
disease gene.

8.   A method according to claim 1 comprising identifying a first
20   gene that enhances or increases activity or effect of the second
gene.

9.   A method according to claim 1 comprising identifying a test
substance that lessens activity or effect of the second gene or
25   said treatment.

10.   A method according to claim 9 wherein the second gene is a
disease gene.

46

11. A method according to claim 1 comprising identifying a test substance that enhances activity or effect of the second gene or said treatment.

5   12. A method according to claim 1 wherein the eye-specific promoter is inducible.

13. A method according to claim 12 wherein the eye-specific promoter is selected from the group consisting of ath5, opsin,

10   rhodopsin, nocturnin, PAX6 or Brn3 promoter, or a specific phototransduction cascade promoter.

14. A method according to any one of claims 1 to 13 wherein the second gene is a disease gene and is selected from the group

15   consisting of human SMN, a truncated form of SMN, wild type or mutant huntingtin, wild type or mutant SOD-1, TNF alpha, wildtype or mutant alpha synuclein, wild type or mutant rhodopsin, wild type or mutant ELOVL4 or wild type or mutant presenilin-1 or -2.

20   15. A method according to claim 1 comprising identifying a first gene that is a suppressor gene of a second gene and in addition to performing the steps set out in claim 1 providing the suppressor gene or a gene product encoded by the suppressor gene in a composition comprising at least one additional component.

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16. A method according to claim 15 further comprising in addition to performing the steps set out in claim 15 providing the suppressor gene or a gene product encoded by the suppressor gene in a screening system for assaying ability of a test

30   substance to affect activity of the suppressor gene or the gene product encoded by the suppressor gene.

17. A method according to claim 16 further comprising in addition to the performing the steps set out in claim 15 and claim 16 providing a test substance that affects activity of the
5   suppressor gene or the gene product encoded by the suppressor gene in a composition comprising at least one additional component.

18. A method according to claim 1 comprising identifying a test
10   substance that affects activity or effect of a second gene or a treatment, and in addition to performing the steps set out in claim 1 providing the test substance in a composition comprising at least one additional component.

15   19. A method according to any one of claims 1 to 18 wherein the fish is selected from the group consisting of zebrafish, fugu and goldfish.

20. A method according to claim 19 wherein the fish is
20   zebrafish.

21. A fish transgenic for a disease gene under regulatory control of an eye-specific promoter, wherein expression of the disease gene within the eye of the fish affects vision of the
25   fish.

22. A fish according to claim 21 wherein the eye-specific promoter is inducible.

30   23. A fish according to claim 22 wherein the eye-specific promoter is selected from the group consisting of ath5, opsin,

48

rhodopsin, nocturnin, PAX6 or Brn3 promoter, or a specific
phototransduction cascade promoter.

24. A fish according to any one of claims 21 to 23 wherein the
disease gene is selected from the group consisting of human SMN,
a truncated form of SMN, wild type or mutant huntingtin, wild
type or mutant SOD-1, TNF alpha, wildtype or mutant alpha
synuclein, wild type or mutant rhodopsin, wild type or mutant
ELOVL4 or wild type or mutant presenilin-1 or -2.

25. A fish according to any one of claims 21 to 24 wherein the
fish is selected from the group consisting of zebrafish, fugu and
goldfish.

26. A fish according to claim 25 wherein the fish is a
zebrafish.

27. Use of a fish according to any one of claims 21 to 26 in a
screen for identifying a first gene that affects activity or
effect of a second gene.

28. Use according to claim 27 wherein the first gene is a
suppressor gene of activity or effect of the second gene, wherein
the second gene is a disease gene.

29. Use of a fish according to any one of claims 21 to 26 in a
screen for identifying a test substance that affects activity or
effect of a second gene or a treatment of a fish.

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   C12N15/00   A01K67/027   A61K49/00   C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C12N   A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	Further documents are listed in the continuation of box C.		X	Patent family members are listed in annex.

* Special categories of cited documents :

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
17 April 2003	08/05/2003

Name and mailing address of the ISA	Authorized officer
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